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Research report

Dose-dependent effects of levetiracetam after hypoxia and hypothermia in the neonatal mouse brain

Katja Strasser [a,b], Laura Lueckemann [a,c], Verena Kluever [a], Sinthuya Thavaneetharajah [a], Daniela Hoeber [a], Ivo Bendix [a], Joachim Fandrey [d], Astrid Bertsche [a,e,*], Ursula Felderhoff-Mueser [a]

[a] Department of Pediatrics 1, Neonatology, Un iversity Hospital Essen, University Duisburg, Essen, Germany
[b] Department of Pediatrics, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany
[c] Institute of Medical Psychology and Behavioral Immunobiology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany
[d] Institute of Physiology, University of Duisburg-Essen, Essen, Germany
[e] Department of Women and Child Health, University Hospital for Children and Adolescents, Centre for Pediatric Research Leipzig, Germany

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ABSTRACT

Perinatal asphyxia to the developing brain remains a major cause of morbidity. Hypothermia is currently the only established neuroprotective treatment available for term born infants with hypoxic-ischemic encephalopathy, saving one in seven to eight infants from developing severe neurological deficits. Therefore, additional treatments with clinically applicable drugs are indispensable. This study investigates a potential additive neuroprotective effect of levetiracetam combined with hypothermia after hypoxia-induced brain injury in neonatal mice.

9-day-old C57BL/6-mice (P9) were subjected either to acute hypoxia or room-air. After 90 min of systemic hypoxia (6% O_2), pups were randomized into six groups: 1) vehicle, 2) low-dose levetiracetam (LEV), 3) high-dose LEV, 4) hypothermia (HT), 5) HT combined with low-dose LEV and 6) HT combined with high-dose LEV. Pro-apoptotic factors, neuronal structures, and myelination were analysed by histology and on protein level at appropriate time points. On P28 to P37 long-term outcome was assessed by neurobehavioral testing.

Hypothermia confers acute and long-term neuroprotection by reducing apoptosis and preservation of myelinating oligodendrocytes and neurons in a model of acute hypoxia in the neonatal mouse brain. Low-dose LEV caused no adverse effects after neonatal hypoxic brain damage treated with hypothermia whereas administration of high-dose LEV alone or in combination with hypothermia increased neuronal apoptosis after hypoxic brain injury. LEV in low- dosage had no additive neuroprotective effect following acute hypoxic brain injury.

1. Introduction

Perinatal asphyxia remains a common cause of neonatal death and long-term disability with an incidence of 20 per 1000 live births (Lee et al., 2013; Rennie et al., 2007). Out of those about 2–3 infants suffer from hypoxic-ischemic encephalopathy (HIE) resulting from significant injury to the developing brain (Murray et al., 2010) with a mortality rate of 20%. Survivors are frequently affected by secondary neurological morbidity, including cerebral palsy (15%), severe cognitive delay (11%), seizure disorders (8%), hearing loss (3%), and visual impairment (3%) (Lee et al., 2013; Rennie et al., 2007).

Perinatal hypoxia results in numerous cell-damaging processes such as neuronal cell injury (Blomgren et al., 2006; Yager et al., 1992) and disturbance of myelination (Back et al., 2001). In response to acute hypoxia, selectively vulnerable regions in the developing mouse brain, mainly striatum, ventrobasal thalamus and periventricular zone can undergo continued apoptosis for a prolonged period up to 6–7 days post insult (Trollmann et al., 2014).

Therapeutic hypothermia within the first 6 h of postnatal life has been shown in animal studies and randomized clinical trials to improve acute brain lesions, survival and neurological long term outcome and is now an established therapy effective in mild and moderate hypoxic-ischemic brain injury with a number needed to treat (NNT) from 7 to 8 (Azzopardi et al., 2014; Shankaran et al., 2012; Edwards et al., 2010; Azzopardi et al., 2009; Gressens et al., 2008; Thoresen et al., 1996). 40–50% of cooled newborns, however,

* Correspondence to: Universitätsklinik und Poliklinik für Kinder und Jugendliche, Liebigstraße 20a, 04103 Leipzig, Germany.
E-mail address: Astrid.Bertsche@medizin.uni-leipzig.de (A. Bertsche).

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still suffer from major neurological problems (Jacobs et al. 2013; Tagin et al. 2012; Edwards et al., 2010). Thus, additional treatment strategies are urgently required.

Symptoms associated with perinatal asphyxia (e.g. muscular hypotonia, cardiorespiratory failure, hyperexcitability, seizures) may occur within the first hours post injury which makes pharmacological treatment essential (Sarnat and Sarnat, 1976). As such, about 60% of patients suffering from hypoxic-ischemic encephalopathy (HIE) and treated with hypothermia need anticonvulsant medication because of clinical or electrographic seizures within the first hours post injury (Shah et al., 2014). Conventional anticonvulsive drugs (e.g. Phenobarbital and Phenytoin), however, are reported to be ineffective in 50% of treated newborns (Booth and Evans, 2004; Boylan et al., 2002). Moreover, they experimentally trigger neuronal apoptosis in the immature rodent brain (Stefovska et al., 2008; Bittigau et al., 2002), and may induce cognitive impairment in infants (Holmes et al., 2001; Dessens et al., 2000).

Levetiracetam (LEV), S-α-ethyl-2-oxo-1-pyrrolidine-acetamide, is an anticonvulsive drug of the second generation which has already been approved for clinical treatment of epilepsy in infants older than 4 weeks of age (Beaulieu et al., 2013) and is also proposed for the intervention in neonatal seizures (Neininger et al., 2015; Ramantani et al., 2011; Silverstein and Ferriero, 2008) with little side effects (Radtke, 2001; Piña-Garza et al., 2009). Furthermore, neuroprotective properties of LEV in high-doses up to 1000 mg/kg body weight are described in the adult rodent brain after stroke (Hanon and Klitgaard, 2001), subarachnoid hemorrhage (Wang et al., 2006) and status epilepticus (Mazarati et al., 2004) as well as in the neonatal brain after hypoxic-ischemic injury (Komur et al., 2014; Kilicdag et al., 2012).

Therefore, LEV is a promising therapy to be administered in conjunction with hypothermia after perinatal asphyxia. Whereas the above mentioned studies in the neonatal brain focused on sole treatment with LEV, combined effects with hypothermia have not been investigated. However, to translate findings into clinical practice a combined treatment strategy with therapeutic hypothermia is obligatory because whole body cooling is the current standard therapy in mild to moderate neonatal hypoxic brain injury.

The aim of this study was to investigate acute and long-term effects of treatment with LEV in combination with hypothermia in a neonatal mouse model of mild to moderate hypoxic brain injury.

2. Results

Animals treated with high-dose (70 mg/kg) LEV had to be excluded from the long-term experiment on P12 due to a significant weight loss of over 20% ($p < 0.001$; data not shown), according to the Federal Guidelines for the Care and Use of Laboratory Animals. After treatment with high-dose LEV, animals showed reduced movements and reduced food intake.

We did not observe any significant sex differences between groups at the various time points investigated.

2.1. LEV influences apoptosis after hypoxic injury in the neonatal brain and treatment with hypothermia

Induction of acute systemic hypoxia (90 min, 6% O_2) on P10 resulted in periventricular tissue loss with enlarged lateral ventricles as revealed by Cresyl violet staining (Fig. 1(A)/(B)). Furthermore, apoptotic cell-death is demonstrated by increased number of DNA-fragmented cells and increased density of cleaved caspase-3 positive cells was detected in hypoxic brains (Fig. 1(C)–(F)). These alterations persisted on P60 with significantly enlarged

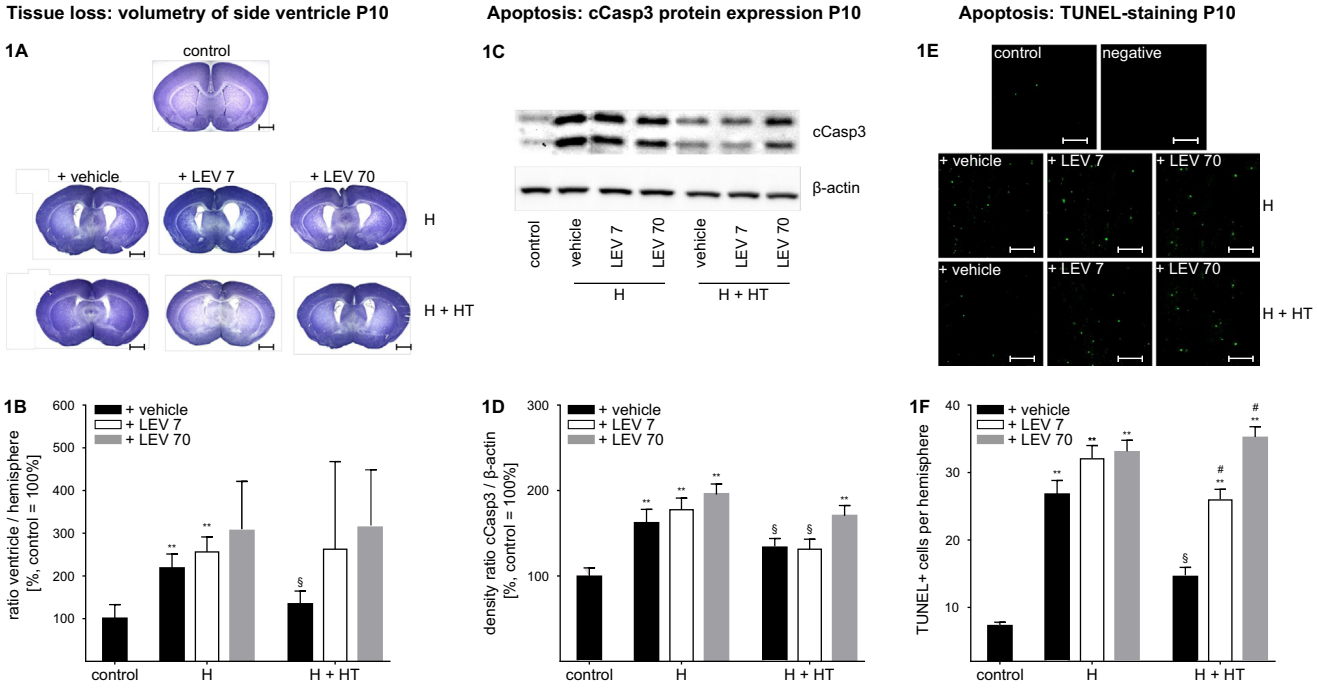

Fig. 1. Hypothermia but not levetiracetam reduces hypoxia-induced brain injury. 9-day-old C57BL/6-mice were subjected to either acute hypoxia or room-air (control). After 90 min hypoxia (6%, H), pups were randomized into six groups: (1) vehicle (0.9% NaCl i.p.), (2) low-dose LEV (7 mg/kg i.p.), (3) high-dose LEV (70 mg/kg i.p.), (4) hypothermia (HT, 4 h 32 °C), (5) HT combined with low-dose LEV and (6) HT combined with high-dose LEV. 24 h after hypoxia brains were analysed for sizes of lateral ventricles via Cresyl-violet-staining. Photographs show representative images of cresyl violet stainings, scale bar is 1 mm (A). The ratio of lateral ventricle sizes to corresponding hemispheres was calculated and from the mean value of control group was set to = 100% (B). Brain extracts of hemispheres were used for western blot analyses of activated Caspase-3; here in a representative Western Blot series of cCasp3 (C). Normalized ratios of cCasp3 signals to signals of β-actin were calculated and the control group was set to 100% (D). DNA fragmented cells were detected via TUNEL-staining in 6 different brain regions; here exemplarily shown for periventricular striatum (E). The sum of positive cells out of all analysed brain regions was calculated (F). Bars represent mean + SD, post-hoc test Bonferroni in (D/F) and Tamhane's T2 in (B). $^{**}p < 0.01$ vs. control group, §$p < 0.05$ vs. hypoxia group, #$p < 0.05$ vs. H + HT. n = 9–10 animals per group for (B) and (F), n = 10–13 animals per group for (D).